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(21) International Application Number: PCT/US93/12464 (22) International Filing Date: 21 December 1993 (21.12.93) (30) Priority Data: 994,650 22 December 1992 (22.12.92) US (60) Parent Application or Grant (63) Related by Continuation US 07/994,650 (CIP) Filed on 22 December 1992 (22.12.92) (71) Applicant (for all designated States except US): THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 300 Lakeside Drive, 22nd floor, Oakland, CA 94612-3550 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): CARSON, Dennis, A. [US/US]; 14824 Vista Del Oceano, Del Mar, CA 92014 (US). WICKS, Ian [AU/AU]; The Walter & Eliza Hall, Institute for Medical Research, P.O. Royal Melbourne Hospital, Melbourne, VIC 3050 (AU).	(74) Agents: HOWELLS, Stacy, L. et al.; Spensley Horn Jubas & Lubitz, 1880 Century Park, Fifth Floor, Los Angeles, CA 90067 (US). (81) Designated States: CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published With international search report.	
(54) Title: METHOD FOR INHIBITION OF CELL ADHESION TO RECEPTORS CONTAINING SELECTINS (57) Abstract <p>A method for inhibition of adhesion of cells to receptors containing selectins in mammals is disclosed whereby an anti-adhesion enzyme is administered to the mammal in a therapeutically effective dosage. In a preferred embodiment, the anti-adhesion enzyme will be administered to treat a chronic or acute inflammatory condition (preferably the latter). In this context, the therapeutically effective dosage will be a dosage sufficient to achieve detectable reduction of the inflammation without substantial toxicity. The enzyme administered will specifically cleave carbohydrate residues which are involved in forming bonds between carbohydrate residues in ligands (typically on leukocytes) which are specific for selectin-containing receptors (typically on endothelial cells), in particular, bonds formed by fucosylated and/or sialylated residues. The enzyme will preferably be a fucosidase or sialidase and is preferably used in recombinant form. Means are also described whereby the activity of the enzyme can be enhanced to perform optimally at pH 7-7.4 (i.e., the pH of plasma). The enzyme may also be modified to extend its <i>in vivo</i> half-life and shelf life. It is expected that the same enzyme will cleave many adhesion residues.</p>		

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METHOD FOR INHIBITION OF CELL ADHESION TO RECEPTORS CONTAINING SELECTINS

BACKGROUND OF THE INVENTION

5 This is a Continuation-In-Part of U.S. Patent Application Serial No. 07/994,650, filed December 22, 1992, now abandoned.

1. *Field of the Invention*

10 The invention relates to a therapeutic method for the inhibition of cell adhesion to cell receptors which contain selectins in mammals, particularly in humans. In particular, it relates to a method of interfering with the adhesion of leukocytes to endothelial cell receptors via specific binding of a selectin in the receptor to its corresponding ligand, such as sialylated and fucosylated ligands. More generally, the invention relates to the prevention of adhesion of any cell via interaction between carbohydrate residues in a cell surface ligand to receptors that contain selectins which will specifically bind
15 such carbohydrate residues.

2. *History of the Invention*

20 Binding of inflammatory cells to the endothelial cells which line blood vessels is a necessary prerequisite to generation of an inflammatory reaction to infection or injury in mammals. Inflammation resulting from the adhesion of neutrophils (phagocytic leukocytes found principally in the blood stream) is known to play a role in acute conditions such as myocardial infarction, stroke, septic shock, anaphylactic reactions to drugs and allergens, and the "post-pump syndrome" which can develop following performance of a cardio-pulmonary bypass. Inflammation is also a major cause of

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disability in chronic conditions such as rheumatoid arthritis, psoriasis, inflammatory bowel disease and chronic obstructive pulmonary disease. Cell adhesion is also important to metastasis of tumors.

5 Recently, researchers have proposed that human inflammatory cells adhere to the endothelium of blood vessels via specific endothelial cell receptor-leukocyte ligand interactions involving cell-type specific carbohydrate structures and linkages. A family of adhesion receptor glycoproteins critical to endothelium binding of leukocytes is known as the selectins. One of the selectins in particular (ELAM-1) is thought to be synthesized by endothelial cells in response to the presence of inflammatory agents.

10 ELAM-1 is brought to the cell surface in its synthesis and serves as a receptor for corresponding carbohydrate adhesion ligands (hereafter, "selectin adhesion ligands") on the cell surface of leukocytes.

Analysis of the adhesion ligands for ELAM-1 and other selectins has indicated that the ligand for ELAM-1 contains carbohydrate residues which are part of a family of

15 sialylated fucosylated polylactosamines, a critical determinant in which is sialyl - 2,3Gal β 1,4 [fucose α 1,3] GlcNAc (hereafter, "sialyl Lewis X"). Sialyl Lewis X and Lewis X are α (1 \rightarrow 3) fucosylated derivatives of polylactosamines found at the non-reducing termini of glycolipids. Glycolipids and glycoproteins that contain α -L-fucose can be hydrolyzed by a lysosomal enzyme known as α -L-fucoside fucosylhydrolase (EC#

20 3.2.1.51), hereafter α -L-fucosidase. Oligosaccharides, gangliosides and glycoproteins which contain sialic acid residues can also be hydrolyzed by a family of enzymes known as the sialidases (EC# 3.2.1.18; also known as neuraminidase).

It has also been suggested that certain tumor cells may metastasize through via cell adhesion events mediated by carbohydrate containing ligand/ selectin containing

25 receptor interactions. It can be expected, therefore, that fucosidases and sialidases will likely also cleave carbohydrate residues involved in metastasis of such tumor cells.

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It is the concept of this invention that, if producible in sufficient quantities of consistent quality, administration of enzymes which will specifically cleave carbohydrate residues in selectin adhesion ligands (hereafter collectively, "anti-adhesion enzymes") in therapeutically effective amounts can block cell adhesion. Such a therapeutic modality would be a significant improvement over current models proposed for blocking cell adhesion to reduce inflammation.

For example, in one current model, cell adhesion is blocked by use of monoclonal antibodies specific to sialyl Lewis X or, as one set of researchers have attempted (Goelz, S.E., *et al.*, *Cell* 63:1349-1356 (1990)) by use of a monoclonal antibody specific to an undefined carbohydrate structure in the selectin ligand. Another approach inhibits cell adhesion by the use of soluble receptors (difucosylated sialyl Lewis X glycolipid or sialyl Lewis X-containing mucin) as competitors for binding of the natural ligand. More recently, researchers have shown that lung vascular endothelial injury associated with inflammation induced in adult respiratory distress syndrome (ARDS) can be minimized in rats by administration of an anti-P-selectin monoclonal antibody (Mulligan, *et al.*, *J.Clin.Invest.* 90:1600-1607 (1992)).

While these approaches have been effective to varying degrees *in vitro*, they appear to have little potential utility for therapeutic purposes because very high concentrations of antibody or soluble receptors must be used to effectively compete with the natural ligand *in vivo*. Further, it is believed that there are a substantial number of selectin adhesion ligands active in inflammatory responses; therefore, a monoclonal antibody or other specific antiligand will not have the broad range of effectiveness of an enzyme specific for carbohydrate moieties in selectin adhesion ligands. A need, therefore, exists for safe and effective cell adhesion inhibitors that target carbohydrate residues in selectin adhesion ligands (particularly the sialylated and fucosylated ligands). The present invention addresses that need.

SUMMARY OF THE INVENTION

The invention is a method for preventing or reducing adhesion of cells mediated by binding of a selectin adhesion ligand to an receptor containing selectin. Preferably, the method involves the administration of a therapeutically effective dosage of an anti-adhesion enzyme (in particular, a sialidase and/or a fucosidase) to a mammal (preferably a human) with acute or chronic inflammation to cleave carbohydrate residues in the ligand, thus preventing adherence of the leukocytes to selectin-containing endothelial cell receptors. It will be appreciated that given the diversity of structure inherent in carbohydrates, other structural components of the selectin ligands on leukocytes may be important to selectin recognition and affinity. However, the data provided in the Examples herein demonstrates that the *in vivo* administration of an anti-adhesion enzyme according to the inventive method results in appreciable reduction in inflammatory responses associated with cell adhesion via receptor/selectin adhesion ligand interactions, particularly acute inflammatory responses.

Because of the prevalence of fucosylated and sialylated selectin adhesion ligands in leukocyte/endothelial cell adhesion events resulting in inflammation, the invention will be principally described in the context of reducing inflammation through administration of the anti-adhesion enzymes of the invention. However, the invention will be understood to not be so limited; i.e., the inventive method for administration of anti-adhesion enzymes may be utilized to inhibit adhesion of any cells wherein the adhesion occurs via formation of a bond between a carbohydrate residue in a cell surface ligand on the cell and a corresponding receptor which contains selectin. A therapeutically effective dosage of the anti-adhesion enzyme will be administered by intravenous, intramuscular or other parenteral routes. Enzymatic activity in the plasma is verified and monitored by monitoring means such as spectrophotometry as well as changes in clinical signs and symptoms.

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Because when administered to a human the enzyme will preferably be a recombinant one of human origin, no immune response should be induced by its administration. Further, because a single enzyme molecule can cleave many different adhesion ligand residues, the potency of the composition is expected to be relatively high, and

5 required dosage relatively low, as compared to prior art proposals for methods of inhibiting cell adhesion by leukocytes.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 depicts the pH activity curve of human α -L-fucosidase.

FIGURE 2 depicts the *in vitro* effect of substantially pure recombinant α -L-fucosidase in varying concentrations on adherence of neutrophils to human umbilical vein endothelial cells.

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FIGURE 3 depicts the effect of sialidase on neutrophil content in lung tissue in rats injected with cobra venom factor (ARDS Model).

DESCRIPTION OF THE PREFERRED EMBODIMENTS

A. Alternative Embodiments of Anti-adhesion Enzymes for Use in the Method of the Invention.

5 The anti-adhesion enzymes of the invention can be extracted from many mammalian tissues, including liver and placental tissues. For purposes of this application, however, the need for a consistent, high volume production source dictates that the recombinant form of the enzymes modified and produced as taught herein will be preferred.

1. Production of recombinant and synthetic anti-adhesion enzymes.

10 (a) Human α -L-fucosidase.

The amino acid sequence shown in SEQ.ID.NO.1 is derived from sequence information obtained through isolation of several human α -L-fucosidase cDNA clones reported by Occhiodoro *et al.* at *Biochem. Biophys. Res. Comm.* 164:439-445 (1989), the disclosure of which is incorporated herein by this reference. Potential
15 glycosylation sites are at nucleotides 754-756, 805-807 and 1147-1149. The full-length nucleotide sequence for α -L-fucosidase is publicly available from the GENBANK molecular sequence database under Accession No. M28099.

As noted above, the fucosidase whose amino acid sequence is depicted in SEQ.ID.NO.1 is preferred for use in the method of the invention for therapy in humans
20 because it is of human origin. It will be appreciated by those skilled in the art, however, that other fucosidases are known and could be of potential therapeutic value in the inventive method, particularly for use in short-term treatment of acute inflammatory conditions where the immunogenicity of the enzyme may not be of as great concern as it would be if used in a treatment regime of longer duration.

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Fucosidase activity in an enzyme to be used in the method of the invention may be determined according to conventional means for determining enzyme activity which are well-known in the art; for example, see Methods of Enzymology (New York Acad. Press, 1981). However, a particularly preferred and well-known method for
5 determining fucosidase activity in an enzyme is disclosed in Zielke, *et al.*, *J. Lab. Clin. Med.* 79:164-169 (1972) and in Aldaheff, *et al.*, *J. Biol. Chem.* 250:7106-7113 (1975). These references are incorporated herein for the purpose of illustrating knowledge in the art regarding determination of fucosidase activity in a sample.

(b) Sialidases.

10 Sialidases have isolated from several vertebrate and microbial sources and cloned and the amino acid/nucleotide sequences reported in publicly accessible molecular sequence databanks such as GENBANK. For example, sialidases cloned from microbial sources include the gene from *Actinomyces viscosus* (GENBANK Accession Nos. S73643, S854402, S69682, X64360, S55807, S55811, S55812, S55814 and
15 S55817); *Salmonella typhimurim* LT2 (GENBANK Accession No.M55342); *Clostridium septicum* (expressed in *E.coli*; GENBANK Accession Nos. X63266, X54369, X54370, X54371, X54372, X54373, X54374, S79270, S79277, M64566); *Clostridium sordellii* G12 (sequence not in public database; reported in Rothe, *et al.*, *J.Gen.Microbiol.* 135:3087-3096 (1989)), and *Clostridium perfringens* (GENBANK Accession No.
20 Y00963; also reported in Roggentin, *et al.*, *FEBS Letters* 238:31-34 (1988)). Sialidase exists in humans but has not previously been cloned; however, there is substantial sequence homology among sialidases which should enable the production of probes for use in cloning human sialidase without undue experimentation (see, e.g., Rothe, *et al.*, *Mol.Gen.Genet.* 226:190-197 (1991) [identification of conserved sequences]; see
25 also, Warner, *et al.*, *Carbohydr.Res.* 215:315-321 (1991) [method for distinguishing mammalian sialidases using inhibition kinetics studies and an unsaturated derivative of N-acetylneuraminic acid]).

With the available information regarding the amino acid and nucleotide sequences of various sialidases, sialidases for use in the inventive method can be readily synthesized or cloned by one of ordinary skill in the art through use of the methods described below without undue experimentation. To that end, each of the above-
5 referenced references, including the related sialidase sequence data from GENBANK and equivalent sequence databases, are incorporated herein as examples of sialidases useful in this invention.

Purified non-human sialidases are also commercially available (from, for example, Sigma Chemical). However, as noted above with respect to the use of fucosidase,
10 use of the human enzyme will be preferred, most preferably in recombinant form.

Sialidase activity in an enzyme to be used in the method of the invention may be determined according to conventional means for determining enzyme activity which are well-known in the art; for example, see Methods of Enzymology (New York Acad. Press, 1981).

15 (c) Methods for producing the recombinant and synthetic anti-adhesion enzymes of the invention.

Methods for producing the anti-adhesion enzymes of the invention are conventional cloning techniques which are well-known in the art. For example, the method of the invention encompasses the use of any catalytically active anti-adhesion enzyme which
20 will cleave bonds between selectins and carbohydrate residues in selectin adhesion ligands, in particular sialidases and fucosidases. It is not intended, therefore, that the invention be considered as limited to the use of the specific enzymes whose amino acid sequences are referred to herein. Rather, functional derivatives of these enzymes and enzymes which are substantially similar in function are encompassed
25 herein. Modifications of these enzymes to form chemical derivatives with additional characteristics, such as extended half-life, are also encompassed by this invention.

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By "functional derivative" is meant the "fragments," "variants," "analogues," or "chemical derivatives" of a molecule. A "fragment" of a molecule, such as any of the anti-adhesion enzyme encoding DNA sequences of the present invention, includes any nucleotide subset of the molecule. A "variant" of such molecule refers to a naturally occurring molecule substantially similar to either the entire molecule, or a fragment thereof. An "analog" of a molecule refers to a non-natural molecule which is substantially similar to either the entire molecule or a fragment thereof.

A molecule is said to be "substantially similar" to another molecule if the sequence of amino acids in both molecules is substantially the same. Substantially similar amino acid molecules will possess a similar biological activity. Thus, provided that two molecules possess a similar activity, they are considered variants as that term is used herein even if one of the molecules contains additional amino acid residues not found in the other, or if the sequence of amino acid residues is not identical. As used herein, a molecule is also said to be a "chemical derivative" of another molecule when it contains additional chemical moieties not normally a part of the molecule. Such moieties may improve the molecule's solubility, absorption, biological half life, etc. The moieties may alternatively decrease the toxicity of the molecule, eliminate or attenuate any undesirable side effect of the molecule, etc. Moieties capable of mediating such effects are disclosed, for example, in *Remington's Pharmaceutical Sciences*, 16th ed., Mack Publishing Co., Easton, Penn. (1980).

Minor modifications of the primary amino acid sequences for the anti-adhesion enzymes of the invention may result in proteins which have substantially equivalent activity as compared to the specific anti-adhesion enzymes described herein. Such modifications may be deliberate, as by site-directed mutagenesis, or may be spontaneous. All of the polypeptides produced by these modifications are included herein as long as the biological activity of an anti-adhesion enzyme (i.e., the ability to specifically cleave bonds between carbohydrate residues and selectins) still exists. Further, deletion of one or more amino acids can also result in a modification of the

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structure of the resultant molecule without significantly altering its biological activity. This can lead to the development of a smaller active molecule which would have broader utility. For example, one can remove amino or carboxy terminal amino acids which may not be required for the enzyme to exert the desired catalytic activity.

- 5 Similarly, a "functional derivative" of a polynucleotide sequence which encodes an anti-adhesion enzyme of the present invention includes "fragments", "variants", or "analogues" of the gene, which may be "substantially similar" in nucleotide sequence, and which encode a molecule possessing similar activity. Thus, as used herein, "anti-adhesion enzyme" includes any functional derivative, fragments, variants, analogues,
10 chemical derivatives which may be substantially similar to the anti-adhesion enzymes described herein and which possess similar activity.

- DNA sequences for use in producing anti-adhesion enzymes other than those identified herein can be obtained by several methods. For example, the DNA can be isolated using hybridization procedures which are well known in the art. These
15 include, but are not limited to: 1) hybridization of probes to genomic or cDNA libraries to detect shared nucleotide sequences; 2) antibody screening of expression libraries to detect shared structural features and 3) synthesis by the polymerase chain reaction (PCR).

- Screening procedures which rely on nucleic acid hybridization make it possible to
20 isolate any gene sequence from any organism, provided the appropriate probe is available. Oligonucleotide probes, which correspond to a part of the sequence encoding the protein in question, can be synthesized chemically. This requires that short, oligopeptide stretches of amino acid sequence must be known. The DNA sequence encoding the protein can be deduced from the genetic code, however, the
25 degeneracy of the code must be taken into account. It is possible to perform a mixed addition reaction when the sequence is degenerate. This includes a heterogeneous mixture of denatured double-stranded DNA. For such screening,

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hybridization is preferably performed on either single-stranded DNA or denatured double-stranded DNA.

5 The development of specific DNA sequences encoding the anti-adhesion enzymes of the invention can also be obtained by: 1) isolation of double-stranded DNA sequences from the genomic DNA; 2) chemical manufacture of a DNA sequence to provide the necessary codons for the polypeptide of interest; and 3) *in vitro* synthesis of a double-stranded DNA sequence by reverse transcription of mRNA isolated from a eukaryotic donor cell. In the latter case, a double-stranded DNA complement of mRNA is eventually formed which is generally referred to as cDNA.

10 DNA sequences encoding the anti-adhesion enzymes can be expressed *in vitro* by DNA transfer into a suitable host cell. "Host cells" are cells in which a vector can be propagated and its DNA expressed. The term also includes any progeny of the subject host cell. It is understood that all progeny may not be identical to the parental cell since there may be mutations that occur during replication. However, 15 such progeny are included when the term "host cell" is used. Methods of stable transfer, in other words when the foreign DNA is continuously maintained in the host, are known in the art.

In the present invention, the genes encoding the anti-adhesion enzymes of the invention may be inserted into a recombinant expression vector. The term 20 "recombinant expression vector" refers to a plasmid, virus or other vehicle known in the art that has been manipulated by insertion or incorporation of the appropriate genetic sequences. Such expression vectors contain a promoter sequence which facilitates the efficient transcription of the inserted genetic sequence of the host.

25 Polynucleotide sequences encoding the anti-adhesion enzymes of the invention can be expressed in either prokaryotes or eukaryotes. Hosts can include microbial, yeast, insect and mammalian organisms. Methods of expressing DNA sequences having

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eukaryotic or viral sequences in prokaryotes are well known in the art. Biologically functional viral and plasmid DNA vectors capable of expression and replication in a host are known in the art. Such vectors are used to incorporate DNA sequences encoding the anti-adhesion enzymes of the invention.

5 Transformation of a host cell with recombinant DNA may be carried out by conventional techniques as are well known to those skilled in the art. Where the host is prokaryotic, such as *E. coli*, competent cells which are capable of DNA uptake can be prepared from cells harvested after exponential growth phase and subsequently treated by the CaCl_2 method by procedures well known in the art. Alternatively,
10 MgCl_2 or RbCl can be used. Transformation can also be performed after forming a protoplast of the host cell or by electroporation.

When the host is a eukaryote, such methods of transfection of DNA as calcium phosphate co-precipitates, conventional mechanical procedures such as microinjection, electroporation, insertion of a plasmid encased in liposomes, or virus
15 vectors may be used. Eukaryotic cells can also be cotransformed with DNA sequences encoding the anti-adhesion enzymes of the invention, and a second foreign DNA molecule encoding a selectable phenotype, such as the herpes simplex thymidine kinase gene. Another method is to use a eukaryotic viral vector, such as simian virus 40 (SV40) or bovine papilloma virus, to transiently infect or transform eukaryotic cells
20 and express the protein. (*Eukaryotic Viral Vectors*, Cold Spring Harbor Laboratory, Gluzman ed., 1982).

The polynucleotides encoding the anti-adhesion enzymes of the invention will preferably contain additional restriction sites to facilitate cloning which are appropriate to the recombinant expression vector to be used, which is Not-I for the preferred
25 vectors. Examples of suitable eukaryotic recombinant expression vectors in addition to those used by Occhiodoro *et al.* are selectable eukaryotic expression vectors available from InVitrogen of San Diego, California (1992 product catalog) such as

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pRc/CMV, pc DNAI/Neo or pRc/RSV, all of which contain a Not-I cloning site. The oligonucleotide for the Not-I enzyme has the sequence 5'-GCGGCCGC-3' and 3'-CGCCGGCG-5'.

5 These latter recombinant expression vectors are preferred for two reasons. First, they contain a neomycin selection marker for stable expression in mammalian cells. Second, use of a eukaryotic vector is generally preferred over prokaryotic vectors because use of a eukaryotic vector will allow for glycosylation of the recombinant enzyme. A prokaryotic recombinant expression vector for transfection into bacteria (preferably *E. coli*) may, however, be used. To this end, suitable prokaryotic
10 expression vectors are available from InVitrogen of San Diego, California and include its pSE280 and pSE380 vector products.

Anti-adhesion enzymes of the invention whose amino acid sequences are known can also be synthesized by the well known solid phase peptide synthesis methods described Merrifield, *J. Am. Chem. Soc.*, 85:2149, (1962), and Stewart and Young,
15 *Solid Phase Peptides Synthesis*, (Freeman, San Francisco, 1969, pp.27-62), using a copoly(styrene-divinylbenzene) containing 0.1-1.0 mMol amines/g polymer. On completion of chemical synthesis, the peptides can be deprotected and cleaved from the polymer by treatment with liquid HF-10% anisole for about 1/4-1 hours at 0°C. After evaporation of the reagents, the peptides are extracted from the polymer with
20 1% acetic acid solution which is then lyophilized to yield the crude material. This can normally be purified by such techniques as gel filtration on Sephadex G-15 using 5% acetic acid as a solvent. Lyophilization of appropriate fractions of the column will yield the homogeneous peptide or peptide derivatives, which can then be characterized by such standard techniques as amino acid analysis, thin layer chromatography, high performance liquid chromatography, ultraviolet absorption spectroscopy,
25 molar rotation, solubility, and quantitated by the solid phase Edman degradation.

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An anti-adhesion enzyme clone having a desirable mutation as described further below may be amplified using a conventional polymerase chain reaction (PCR) and a primer pair corresponding to the 3' and 5' regions of the cDNA. The preferred method of amplification is the overlap extension PCR technique described by Ho, *et al.*, *Gene* 77:51-59 (1989), the disclosure of which is incorporated herein by this reference. Generally, this technique accomplishes site-specific mutagenesis of the clone by utilizing a 3' primer to add the mismatched mutating bases (primer B in the Ho article, which is used with the 5' primer A in the first PCR cycle described). Amplification using the A and B primers yields an AB fragment. A second PCR cycle uses a primer (D) from the 3' end of the gene and a 5' mutated primer (C) complementary to primer B. The resulting amplification product (fragment CD) will overlap the AB fragment. When the AB and CD fragments are denatured, reannealed and amplified using the A and D primers, the resulting fusion product (AD) will contain the full-length cDNA sequence and the desired mutation.

It should be noted that it may be necessary to mutate any site of N-linked glycosylation in the anti-adhesion enzyme polypeptides of the invention to prevent mannosylation of the protein. This will prevent uptake of the recombinant enzyme by monocytes and macrophages that have receptors for mannose-phosphate, thus extending the half-life of the enzyme in a patient's circulation.

The overlap extension PCR technique described above may be used to eliminate the sites for N-linked glycosylation in the anti-adhesion enzyme produced according to the method described herein. For purposes of illustration, the polymerase chain reaction technique may be modified to change 1 to 3 of the asparagines in the sites of N-linked glycosylation to lysines or arginine (e.g., to change the AAT or AAC codons for asparagine to AGA or AGG, the codons for arginine).

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Another suitable approach to modification of the lysines or arginines is described by Shaw in U.S. Patent No. 4,904,584 ("Site-Specific Homogenous Modification of Polypeptides"), the disclosure of which is incorporated herein by this reference for purposes of illustration.

5 If, however, it is determined by assays described above that glycosylation of the enzyme enhances its desired functional activity, the interest in extending the half-life of the enzyme may be outweighed. In that instance, therefore, the sites for N-linked glycosylation will not be modified and a eukaryotic vector will be used to express the anti-adhesion enzyme.

10 Another useful mutation of the recombinant enzyme is one which will raise the pH optimum for the enzyme's activity from pH 5-5.5 to pH 7-7.4, the normal pH for human plasma (see, e.g., FIGURE 4 for the normal pH activity curve for α -L-fucosidase in human serum [as determined at varying pH values in citrate-phosphate buffers for 30 minutes at 37°C]). This change to enhance the enzyme's activity in more alkaline
15 environments is achieved by separately mutating single charged amino acids selected from the group consisting of lysine, arginine, histidine, glutamic and aspartic acid. Presently, the identity of the amino acid whose mutation will best enhance the activity of each of the anti-adhesion enzymes of the invention at pH 7-7.4 is not known, but can be systematically determined without undue experimentation by site-specific
20 mutagenesis wherein a single charged amino acid will be substituted for with a different amino acid the product expressed as described above. An assay for enzyme activity at the desired pH will be performed according to the method disclosed in Zielke, *et al.*, *J. Lab. Clin. Med.* 79:164-169 (1972), and the process repeated performing different non-cumulative mutations until the optimum pH for the desired
25 enzyme activity has been raised to pH 7-7.4.

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Once produced, the anti-adhesion enzyme will be purified by means well-known in the art to a preferred specific activity. For example, with respect to fucosidase, purification is best achieved by affinity chromatography using agarose to which aminocaproyl fucosamine has been coupled (suitable columns for use in this procedure are available from Sigma Chemical of St. Louis, Missouri or from Miles Laboratory of Naperville, Illinois).

More specifically, the tissue extract, transformed cell line supernatants, transformed bacterial lysates, or other source of the synthetic or recombinant fucosidase may be applied to an affinity column and washed with 10 mM phosphate buffer (pH 5.5) until all protein has been eluted. The column is then washed again with the phosphate buffer supplemented with 50 mM fucose available from Sigma) to elute the native or recombinant fucosidase, which is then concentrated using an ultrafiltration device (such as the ultrafiltration apparatus and microconcentrators available from Amicon of Danvers, Massachusetts) to a preferred concentration of 1.5 to 2.0 mg of enzyme per milliliter of buffer. Purity is confirmed according to means well known in the art for performance of SDS-polyacrylamide gel electrophoresis. All of the procedures described are performed at 2°-4°C and may be adapted for the purification of sialidases by one of ordinary skill in the art without undue experimentation.

The purified anti-adhesion enzyme will preferably have a specific activity of 25,000 - 30,000 units/mg of protein, wherein one unit (U) of enzyme activity is equivalent to the amount of enzyme which will hydrolyze 1 nmole of P-nitrophenyl- α -L-fucopyranoside or sialic acid substrate (available from Sigma) per minute at 37°C.

B. Preparation of Pharmacologically Effective Compositions of
Anti-adhesion Enzymes

Once the desired anti-adhesion enzyme has been produced and purified as described above, the enzyme is suspended in a buffer suitable for administration to humans, preferably isotonic phosphate buffered saline (pH 7.4) at a concentration of 1 to 10 mg enzyme/ml buffer, not to exceed 100 ml of buffer. The buffer may also be supplemented with stabilizers and/or anti-bacterial agents well known to those skilled in the art to maintain purity and extend shelf life of the anti-adhesion enzyme suspension.

In a preferred embodiment of the anti-adhesion enzyme composition, the enzyme will be modified to delay its clearance from the patient's bloodstream via the kidneys, thus extending the patient's exposure to the enzyme and reducing the number of times that the composition will have to be administered for effective treatment. To this end, the enzyme may be covalently attached to activated polyethylene glycol (PEG), which couples to the lysines and arginines in the protein.

Activated PEG is available from, for example, Sigma Chemical, in five different forms; an exemplary form for use in this embodiment is methoxypolyethylene glycol-succinimidyl succinate. Whichever form of PEG is chosen, its selection should be made with a view toward chemically coupling at least 50% of the lysines and arginines in the protein.

The actual percentage of these amino acids which will be coupled in the final enzyme product will be that percentage which will help to maintain availability of the enzyme to the patient without destroying the catalytic activity of the enzyme. Generally, it can be expected that at least 50% of these amino acids will be coupled with PEG to avoid clearance of the enzyme by the patient's kidneys; the degree to which the useful life of the enzyme can be extended by coupling additional lysines and arginines to PEG

will depend on whether additional coupling significantly reduces enzyme activity. The point at which enzyme activity is significantly reduced can be determined by the assay procedures for enzyme activity described above (see, e.g., Aldaheff, *et al.*, *J. Biol. Chem.* 250:7106-7113 (1975)).

5 Coupling of the lysines and arginines in the enzyme product to PEG may be achieved by use of several methods known to those skilled in the art (see, e.g., Savoca, *et al.*, *Biochem. Biophys. Acta.* 578:47-53 (1979) and methods used in U.S. Patent 4,766,106). A suitable adaptation of these methods to conjugation of the anti-adhesion enzymes of the invention would be performed as follows. Approximately 25
10 mg of purified recombinant enzyme is dissolved in 5 ml of 50 mM phosphate buffered saline (pH 7.2). The dissolved enzyme is then incubated with approximately 500 mg of activated polyethyleneglycol (such as methoxypolyethylene glycol-succinimide succinate, available from Sigma), then isolated by means known in the art (see, e.g., Abuchowski *et al.*, "Enzymes as Drugs", John Wiley and Sons (1981)).

15 **C. Clinical Administration of the Anti-adhesion Enzyme Composition.**

The anti-adhesion enzyme composition will be administered to the mammal in a therapeutically effective dosages, preferably by intra-arterial infusion. In this context, a "therapeutically effective dosage" refers to that amount of anti-adhesion enzyme which will produce a detectable reduction in inflammation without substantial toxicity
20 to the patient. Generally, this dosage will comprise about 0.6 to 234 U/kg of the patient's body weight.

Typically, the infusion will be performed for an extended period of up to about 4 hours, although those of ordinary skill in the clinical art will know or be able to readily determine how to adjust the course of treatment to suit the therapy needs of
25 particular patients. Administration of this dosage will preferably be repeated at intervals of two times per week to once a month, depending on the patient's

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presenting condition and responsiveness to the therapy. Enzymatic activity in the plasma is verified and monitored by spectrophotometry according to means well-known in the art; changes in clinical signs and symptoms will provide a further basis for assessment of functional enzyme activity.

- 5 Based on the role of leukocytes in endothelial cell activation, it is expected that the anti-adhesion enzyme suspension will be effective in therapy for acute inflammatory conditions associated with septic and endotoxin shock, post-pump syndrome following cardiopulmonary bypass, the acute rejection of transplanted organs, reprofusion injury following myocardial infarction or stroke and peripheral vascular
10 injuries. It is also expected to be of therapeutic value in treatment of chronic inflammation associated with rheumatoid arthritis, inflammatory bowel disease, psoriasis, multiple sclerosis and inflammatory iritis.

- 15 Based on *in vitro* exposure of tumor cells having metastatic capability (rat mammary adenocarcinoma cell line R13762) to bovine fucosidase (see data reported in Wright, *et al. J. Cell. Biochem.* 37:49-59, (1988)) it is also expected that the human fucosidase composition and, possibly, the sialidase composition disclosed herein will be of value in limiting metastasis of tumor cells including, but not limited to, those associated with cancers of the breast, colon, prostate, lung, stomach and pancreas.

- 20 It will be appreciated by those skilled in the art that there may be various ways to practice this invention. These examples should, therefore, be regarded as illustrative rather than exhaustive of those possibilities.

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EXAMPLE I**PROTOCOL FOR MEASUREMENT OF *IN VIVO* Anti-adhesion
RESPONSE TO FUCOSIDASE COMPOSITION IN RABBIT MODEL**

5 A substantially pure α -L-fucosidase composition was prepared as discussed above without coupling to PEG or mutation to enhance optimal pH activity or to prevent mannosylation. In this context, "substantially pure" refers to a fucosidase composition which consists essentially of catalytically active fucosidase, but may also contain catalytically active sialidase. The pRc/CMV or pcDNA1/Neo eukaryotic expression vectors available from InVitrogen of San Diego, California were used to express the
10 enzyme.

The fucosidase composition was injected in a dosage of 0.01-0.1 mg/kg of body weight into New Zealand white rabbits of 2-3 kg body weight. One hour later, carrageenan (available from Sigma in a preparation of 2.5 mg carrageenan/0.25 ml water) was injected into the pleural cavity of each rabbit. Four hours following the
15 carrageenan injection, the rabbits were sacrificed and the plural cavity opened. The plural exudate was carefully removed by pipetting and assayed for total volume of exudate (in milliliters) and for total cellular influx.

The effectiveness of the suspension is demonstrated by a $\geq 25\%$ reduction in total exudate volume in the pleural cavity and in cellular influx, as compared to controls
20 injected only with carrageenan. This assay can be performed at with any site of extravascular inflammation, such as in the skin of the ear. It should be noted, however, that use of rats in lieu of rabbits or other mammalian models is not recommended because fucose apparently does not play a significant role in rat inflammation responses.

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EXAMPLE II**IN VITRO INHIBITION OF NEUTROPHIL ADHERENCE TO HUMAN
UMBILICAL VEIN ENDOTHELIAL CELLS**

5 Human umbilical vein endothelial cells (HUVEC) available from Clonetics of San Diego, California were propagated in microtiter plates in EGMUV medium (also available from Clonetics) which were supplemented with 2% fetal bovine serum and used during the first three passages. To increase their adhesiveness, the HUVEC cells were treated with recombinant interleukin-1 (IL-1) (R&D Systems, Minneapolis, MN) at a concentration of 5 ng/ml for four hours prior to analysis.

10 Neutrophils were isolated from human peripheral blood by Ficoll-Hypaque sedimentation according to means well known in the art. The cell pellet was treated for 10 seconds with water to lyse erythrocytes, and then was washed two times in Hank's balanced salt solution (HBSS) supplemented with 1% human plasma. The isolated neutrophils were incubated with bovine kidney fucosidase at the concentrations
15 indicated in FIGURE 2 for one hour in HBSS with 1% autologous plasma. The cells were then washed and then added at a density of 200,000 cells/ml to the HUVEC monolayers. After 45 minutes at 37°C, unadhered neutrophils were removed gently with a pipette followed by rinsing with warm HBSS containing 1% plasma. Then 0.1% Triton X-100 is added to the cultures to lyse the neutrophils.

20 After an overnight incubation, released neutrophil myeloperoxidase was measured spectrophotometrically by the oxidation of diaminobenzidine with H₂O₂ in the presence of the enzyme, using a commercial assay (the kit for which is available from Sigma, St. Louis). Since myeloperoxidase is present in neutrophils and not endothelial cells, the total enzyme level is directly proportional to the number of
25 adherent neutrophils in each culture. As indicated in FIGURE 2, pretreatment of the neutrophils with the fucosidase composition blocked their ability to adhere to HUVEC.

EXAMPLE III
PREVENTION OF NEUTROPHIL ENTRY INTO LUNG TISSUE
IN AN ADULT RESPIRATORY DISTRESS SYNDROME MODEL

- 5 A sialidase composition of microbial origin (*Clostridium*) was prepared as described elsewhere above. To create a model for the acute inflammatory condition known as adult respiratory distress syndrome (ARDS), "COBRA VENOM FACTOR" (CVF), a tradenamed product of Calbiochem of San Diego, was administered to rats as follows. In this model of ARDS, CVF activates the complement system. The resulting formation of the complement factor C5A induces neutrophil activation. The activated
- 10 neutrophils accumulate in the lung vasculature, causing tissue damage. CVF was diluted in phosphate buffered saline (PBS) to 32 μ /ml and dialyzed overnight at 4°C in PBS. The final concentration of the CVF was 22.8 μ /ml. The sialidase was resuspended in PBS to a volume of 50 μ /ml and injected into the rat's tails in volume of 200 μ l of PBS. Control rats received injections of 0.5 ml PBS.
- 15 After 80 minutes, secondary injections were made of either PBS (control rats) or 11.4 units of CVF. Thirty minutes later, rats were anaesthetized with an intraperitoneal injection of ketamine hydrochloride. Rat lung vasculature was then perfused with 10 ml PBS injected in the right ventricle with the inferior vena cava and superior vena cava clamped.
- 20 Lung tissue was dissected out with a specimen fixed in formalin for histological study and a second specimen placed in PBS for myeloperoxidase activity assay. Rat lungs in PBS were frozen at -20°C for 2 days. After 2 days, rat lung specimens were thawed in a 37 °C circulating water bath. Approximately 500 mg of lung tissue was minced to fine pieces approximately 1 mm² using sterile scalpel and forceps. Lung
- 25 pieces were then sonicated at a setting of 4.5 on a microtip sonicator for 45, 30 and 10 second intervals. After sonication, 1.5 ml aliquots were transferred to microcentrifuge tubes and centrifuged at 4°C for 3 minutes at 16,000 RCF.

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Supernatants were transferred to new microcentrifuge tubes and assayed for myeloperoxidase activities as described elsewhere above.

5 The result of this assay demonstrates that the rats which received the sialidase injections were able to fend off the neutrophil entry into lung tissue which characterizes the inflammatory response in ARDS. The data from the sialidase injected mice and the control mice are shown in FIGURE 3.

It will be appreciated that modifications to this invention, which is defined by the appended claims, may be made without departing from its spirit or scope.

SUMMARY OF SEQUENCES

SEQUENCE ID NO 1 is the polynucleotide sequence which will encode human α -L-fucosidase, including the signal peptide (bases 19 to 84) and the mature protein (bases 85 to 1401).

- 5 SEQUENCE ID NO 2 is the deduced amino acid sequence for human α -L-fucosidase, including the signal peptide and mature protein.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: The Regents of the University of California, *et al.*

5 (ii) TITLE OF INVENTION: METHOD FOR INHIBITION OF CELL ADHESION TO
RECEPTORS CONTAINING SELECTINS

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:

10 (A) ADDRESSEE: SPENSLEY HORN JUBAS & LUBITZ
(B) STREET: 1880 CENTURY PARK EAST, FIFTH FLOOR
(C) CITY: LOS ANGELES
(D) STATE: CALIFORNIA
(E) COUNTRY: US
15 (F) ZIP: 90067

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
20 (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: PCT
(B) FILING DATE: 21-DEC-1993
(C) CLASSIFICATION:

25 (viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: HOWELLS, STACY L.
(B) REGISTRATION NUMBER: 34,842
(C) REFERENCE/DOCKET NUMBER: FD-2929

(ix) TELECOMMUNICATION INFORMATION:

30 (A) TELEPHONE: (619) 455-5100
(B) TELEFAX: (619) 455-5110

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 2035 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

(B) CLONE: ALPHA-L-FUCOSIDASE

10 (ix) FEATURE:

- (A) NAME/KEY: CDS
 (B) LOCATION: 19..1401

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

15	GAATTCCGGG CTCCGGGG ATG AGG TCG CGG CCG GCG GGT CCC GCG CTG TTG	51
	Met Arg Ser Arg Pro Ala Gly Pro Ala Leu Leu	
	1 5 10	
	CTG CTG CTG CTC TTC CTC GGA GCG GCC GAG TCG GTG CGT CGG GCC CAG	99
	Leu Leu Leu Leu Phe Leu Gly Ala Ala Glu Ser Val Arg Arg Ala Gln	
	15 20 25	
20	CCT CCG CGC CGC TAC ACC CCA GAC TGG CCG AGC CTG GAT TCT CGG CCG	147
	Pro Pro Arg Arg Tyr Thr Pro Asp Trp Pro Ser Leu Asp Ser Arg Pro	
	30 35 40	
	CTG CCG GCC TGG TTC GAC GAA GCC AAG TTC GGG GTG TTC ATC CAC TGG	195
	Leu Pro Ala Trp Phe Asp Glu Ala Lys Phe Gly Val Phe Ile His Trp	
25	45 50 55	
	GGC GTG TTC TCG GTG CCC GCC TGG GGC AGC GAG TGG TTC TGG TGG CAC	243
	Gly Val Phe Ser Val Pro Ala Trp Gly Ser Glu Trp Phe Trp Trp His	
	60 65 70 75	
	TGG CAG GGC GAG GGG CGG CCG CAG TAC CAG CGC TTC ATG CGC GAC AAC	291
30	Trp Gln Gly Glu Gly Arg Pro Gln Tyr Gln Arg Phe Met Arg Asp Asn	
	80 85 90	

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	TAC	CCG	CCC	GGC	TTC	AGC	TAC	GCC	GAC	TTC	GGA	CCG	CAG	TTC	ACT	GCG	339
	Tyr	Pro	Pro	Gly	Phe	Ser	Tyr	Ala	Asp	Phe	Gly	Pro	Gln	Phe	Thr	Ala	
			95						100					105			
	CGC	TTC	TTC	CAC	CCG	GAG	GAG	TGG	GCC	GAC	CTC	TTC	CAG	GCC	GCG	GGC	387
5	Arg	Phe	Phe	His	Pro	Glu	Glu	Trp	Ala	Asp	Leu	Phe	Gln	Ala	Ala	Gly	
		110						115					120				
	GCC	AAG	TAT	GTA	GTT	TTG	ACG	ACA	AAG	CAT	CAC	GAA	GGC	TTC	ACA	AAC	435
	Ala	Lys	Tyr	Val	Val	Leu	Thr	Thr	Lys	His	His	Glu	Gly	Phe	Thr	Asn	
		125					130					135					
10	TGG	CCG	AGT	CCT	GTG	TCT	TGG	AAC	TGG	AAC	TCC	AAA	GAC	GTG	GGG	CCT	483
	Trp	Pro	Ser	Pro	Val	Ser	Trp	Asn	Trp	Asn	Ser	Lys	Asp	Val	Gly	Pro	
	140					145					150				155		
	CAT	CGG	GAT	TTG	GTT	GGT	GAA	TTG	GGA	ACA	GCT	CTC	CGG	AAG	AGG	AAC	531
15	His	Arg	Asp	Leu	Val	Gly	Glu	Leu	Gly	Thr	Ala	Leu	Arg	Lys	Arg	Asn	
				160						165					170		
	ATC	CGC	TAT	GGA	CTA	TAC	CAC	TCA	CTC	TTA	GAG	TGG	TTC	CAT	CCA	CTC	579
	Ile	Arg	Tyr	Gly	Leu	Tyr	His	Ser	Leu	Leu	Glu	Trp	Phe	His	Pro	Leu	
				175					180					185			
	TAT	CTA	CTT	GAT	AAG	AAA	AAT	GGC	TTC	AAA	ACA	CAG	CAT	TTT	GTC	AGT	627
20	Tyr	Leu	Leu	Asp	Lys	Lys	Asn	Gly	Phe	Lys	Thr	Gln	His	Phe	Val	Ser	
			190					195					200				
	GCA	AAA	ACA	ATG	CCA	GAG	CTG	TAC	GAC	CTT	GTT	AAC	AGC	TAT	AAA	CCT	675
	Ala	Lys	Thr	Met	Pro	Glu	Leu	Tyr	Asp	Leu	Val	Asn	Ser	Tyr	Lys	Pro	
		205					210					215					
25	GAT	CTG	ATC	TGG	TCT	GAT	GGG	GAG	TGG	GAA	TGT	CCT	GAT	ACT	TAC	TGG	723
	Asp	Leu	Ile	Trp	Ser	Asp	Gly	Glu	Trp	Glu	Cys	Pro	Asp	Thr	Tyr	Trp	
	220					225					230					235	

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	AAC	TCC	ACA	AAT	TTT	CTT	TCA	TGG	CTC	TAC	AAT	GAC	AGC	CCT	GTC	AAG	771
	Asn	Ser	Thr	Asn	Phe	Leu	Ser	Trp	Leu	Tyr	Asn	Asp	Ser	Pro	Val	Lys	
					240					245					250		
	GAT	GAG	GTG	GTA	GTA	AAT	GAC	CGA	TGG	GGT	CAG	AAC	TCT	TCC	TGT	CAC	819
5	Asp	Glu	Val	Val	Val	Asn	Asp	Arg	Trp	Gly	Gln	Asn	Ser	Ser	Cys	His	
				255					260					265			
	CAT	GGA	GGA	TAC	TAT	AAC	TGT	GAA	GAT	AAA	TTC	AAG	CCA	CAG	AGC	TTG	867
	His	Gly	Gly	Tyr	Tyr	Asn	Cys	Glu	Asp	Lys	Phe	Lys	Pro	Gln	Ser	Leu	
			270					275					280				
10	CCA	GAT	CAC	AAG	TGG	GAG	ATG	TGC	ACC	AGC	ATT	GAC	AAG	TTT	TCC	TGG	915
	Pro	Asp	His	Lys	Trp	Glu	Met	Cys	Thr	Ser	Ile	Asp	Lys	Phe	Ser	Trp	
		285					290					295					
	GGC	TAT	CGT	CGT	GAC	ATG	GCA	TTG	TCT	GAT	GTT	ACA	GAA	GAA	TCT	GAA	963
	Gly	Tyr	Arg	Arg	Asp	Met	Ala	Leu	Ser	Asp	Val	Thr	Glu	Glu	Ser	Glu	
15	300					305					310				315		
	ATC	ATT	TCG	GAA	CTG	GTT	CAG	ACA	GTA	AGT	TTG	GGA	GGC	AAC	TAT	CTT	1011
	Ile	Ile	Ser	Glu	Leu	Val	Gln	Thr	Val	Ser	Leu	Gly	Gly	Asn	Tyr	Leu	
					320					325					330		
	CTG	AAC	ATT	GGA	CCA	ACT	AAA	GAT	GGA	CTG	ATT	GTT	CCC	ATC	TTC	CAA	1059
20	Leu	Asn	Ile	Gly	Pro	Thr	Lys	Asp	Gly	Leu	Ile	Val	Pro	Ile	Phe	Gln	
				335					340					345			
	GAA	AGG	CTT	CTT	GCT	GTT	GGG	AAA	TGG	CTG	AGC	ATC	AAT	GGG	GAG	GCT	1107
	Glu	Arg	Leu	Leu	Ala	Val	Gly	Lys	Trp	Leu	Ser	Ile	Asn	Gly	Glu	Ala	
			350					355					360				
25	ATC	TAT	GCC	TCC	AAA	CCA	TGG	CGG	GTG	CAA	TGG	GAA	AAG	AAC	ACA	ACA	1155
	Ile	Tyr	Ala	Ser	Lys	Pro	Trp	Arg	Val	Gln	Trp	Glu	Lys	Asn	Thr	Thr	
		365					370					375					
	TCT	GTA	TGG	TAT	ACC	TCA	AAG	GGA	TCG	GCT	GTT	TAT	GCC	ATT	TTT	CTG	1203
	Ser	Val	Trp	Tyr	Thr	Ser	Lys	Gly	Ser	Ala	Val	Tyr	Ala	Ile	Phe	Leu	
30	380					385				390					395		
	CAC	TGG	CCA	GAA	AAT	GGA	GTC	TTA	AAC	CTT	GAA	TCC	CCC	ATA	ACT	ACC	1251
	His	Trp	Pro	Glu	Asn	Gly	Val	Leu	Asn	Leu	Glu	Ser	Pro	Ile	Thr	Thr	
					400					405					410		

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	TCA ACT ACA AAG ATA ACA ATG CTG GGA ATT CAA GGA GAT CTG AAG TGG	1299
	Ser Thr Thr Lys Ile Thr Met Leu Gly Ile Gln Gly Asp Leu Lys Trp	
	415 420 425	
	TCC ACA GAT CCA GAT AAA GGT CTC TTC ATC TCT CTA CCC CAG TTG CCA	1347
5	Ser Thr Asp Pro Asp Lys Gly Leu Phe Ile Ser Leu Pro Gln Leu Pro	
	430 435 440	
	CCC TCT GCT GTC CCC GCA GAG TTT GCT TGG ACT ATA AAG CTG ACA GGA	1395
	Pro Ser Ala Val Pro Ala Glu Phe Ala Trp Thr Ile Lys Leu Thr Gly	
	445 450 455	
10	GTG AAG TAATCATTG AGTGCAAGAA GAAAGAGGCG CTGCTCACTG TTTTCCTGCT	1451
	Val Lys	
	TCAGTTTTTC TCTTATAGTA CCATCACTAT AATCAACGAA CTTCTCTTCT CCACCCAGAG	1511
	ATGGCTTTTC CAACACATTT TAATTAAAGG AACTGAGTAC ATTACCCTGA TGTCTAAATG	1571
15	GACCAAAGAT CTGAGATCCA TTGTGATTAT ATCTGTATCA GGTCAGCAGA AGAAGGAACT	1631
	GAGCAGTTGA ACTCTGAGTT CATCAATTCT AATATTTGGA AATTATCTAC AATGGAATCT	1691
	TCCCTCTGTT CTCTGATAAC CTAATTGCTT ACTCAATGCC TTTAAGCCAA GTCACCCTGT	1751
	TGCCTATGGG AGGAGGTGGA AGGATTGCGC AAGCTCAACC ACATGCTATT TAGTTAGCAT	1811
	CAGTTGTCAC CAACAGTCTT TCTGCAAAGG GCAGGAGAGC TTTGGGGGAA AGGAAAAGGC	1871
20	TTACCAGGCT GCTATGGTCA ACTCTTCAGA AATTTTCAGA GCAATCTAAA AGCGCCAAAA	1931
	TTGGCTATGT TTACAGTGAT ACTATTAAGA AAATGAATGT GATTCTGCTC TGTCTTTTAA	1991
	AGTATGATCA AATAAAAAAT TTGTACATCA CAATCATTTT TAGC	2035

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 461 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```

Met Arg Ser Arg Pro Ala Gly Pro Ala Leu Leu Leu Leu Leu Phe
 1             5             10             15

10  Leu Gly Ala Ala Glu Ser Val Arg Arg Ala Gln Pro Pro Arg Arg Tyr
      20             25             30

Thr Pro Asp Trp Pro Ser Leu Asp Ser Arg Pro Leu Pro Ala Trp Phe
      35             40             45

15  Asp Glu Ala Lys Phe Gly Val Phe Ile His Trp Gly Val Phe Ser Val
      50             55             60

Pro Ala Trp Gly Ser Glu Trp Phe Trp Trp His Trp Gln Gly Glu Gly
 65             70             75             80

Arg Pro Gln Tyr Gln Arg Phe Met Arg Asp Asn Tyr Pro Pro Gly Phe
      85             90             95

20  Ser Tyr Ala Asp Phe Gly Pro Gln Phe Thr Ala Arg Phe Phe His Pro
      100            105            110

Glu Glu Trp Ala Asp Leu Phe Gln Ala Ala Gly Ala Lys Tyr Val Val
      115            120            125

25  Leu Thr Thr Lys His His Glu Gly Phe Thr Asn Trp Pro Ser Pro Val
      130            135            140

Ser Trp Asn Trp Asn Ser Lys Asp Val Gly Pro His Arg Asp Leu Val
 145            150            155            160

Gly Glu Leu Gly Thr Ala Leu Arg Lys Arg Asn Ile Arg Tyr Gly Leu
      165            170            175

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	Tyr	His	Ser	Leu	Leu	Glu	Trp	Phe	His	Pro	Leu	Tyr	Leu	Leu	Asp	Lys	
				180					185					190			
	Lys	Asn	Gly	Phe	Lys	Thr	Gln	His	Phe	Val	Ser	Ala	Lys	Thr	Met	Pro	
		195						200					205				
5	Glu	Leu	Tyr	Asp	Leu	Val	Asn	Ser	Tyr	Lys	Pro	Asp	Leu	Ile	Trp	Ser	
	210						215					220					
	Asp	Gly	Glu	Trp	Glu	Cys	Pro	Asp	Thr	Tyr	Trp	Asn	Ser	Thr	Asn	Phe	
	225					230					235					240	
	Leu	Ser	Trp	Leu	Tyr	Asn	Asp	Ser	Pro	Val	Lys	Asp	Glu	Val	Val	Val	
10					245					250					255		
	Asn	Asp	Arg	Trp	Gly	Gln	Asn	Ser	Ser	Cys	His	His	Gly	Gly	Tyr	Tyr	
				260					265					270			
	Asn	Cys	Glu	Asp	Lys	Phe	Lys	Pro	Gln	Ser	Leu	Pro	Asp	His	Lys	Trp	
		275						280					285				
15	Glu	Met	Cys	Thr	Ser	Ile	Asp	Lys	Phe	Ser	Trp	Gly	Tyr	Arg	Arg	Asp	
	290						295					300					
	Met	Ala	Leu	Ser	Asp	Val	Thr	Glu	Glu	Ser	Glu	Ile	Ile	Ser	Glu	Leu	
	305					310					315					320	
	Val	Gln	Thr	Val	Ser	Leu	Gly	Gly	Asn	Tyr	Leu	Leu	Asn	Ile	Gly	Pro	
20					325					330					335		
	Thr	Lys	Asp	Gly	Leu	Ile	Val	Pro	Ile	Phe	Gln	Glu	Arg	Leu	Leu	Ala	
				340					345					350			
	Val	Gly	Lys	Trp	Leu	Ser	Ile	Asn	Gly	Glu	Ala	Ile	Tyr	Ala	Ser	Lys	
		355						360					365				
25	Pro	Trp	Arg	Val	Gln	Trp	Glu	Lys	Asn	Thr	Thr	Ser	Val	Trp	Tyr	Thr	
	370						375						380				
	Ser	Lys	Gly	Ser	Ala	Val	Tyr	Ala	Ile	Phe	Leu	His	Trp	Pro	Glu	Asn	
	385				390						395					400	
	Gly	Val	Leu	Asn	Leu	Glu	Ser	Pro	Ile	Thr	Thr	Ser	Thr	Thr	Lys	Ile	
30					405					410					415		

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Thr Met Leu Gly Ile Gln Gly Asp Leu Lys Trp Ser Thr Asp Pro Asp
420 425 430

Lys Gly Leu Phe Ile Ser Leu Pro Gln Leu Pro Pro Ser Ala Val Pro
435 440 445

5 Ala Glu Phe Ala Trp Thr Ile Lys Leu Thr Gly Val Lys
450 455 460

CLAIMS

1. A method for inhibition of adhesion of cells to receptors containing selectins in a mammal comprising:

5 administration to the mammal of a pharmaceutically acceptable composition containing a therapeutically effective dosage of at least one anti-adhesion enzyme, which enzyme will specifically cleave a carbohydrate residue in a selectin adhesion ligand for selectin-containing receptors on endothelial cells.

2. The method according to Claim 1 wherein the residue is sialylated.
3. The method according to Claim 2 wherein the anti-adhesion enzyme comprises a sialidase.
4. The method according to Claim 1 wherein the residue is fucosylated.
5. The method according to Claim 1, wherein the anti-adhesion enzyme comprises a fucosidase.
6. The method according to Claim 1, wherein the composition contains sialidase and fucosidase.
7. The method according to Claim 3, wherein the sialidase is of human origin.
8. The method according to Claim 5, wherein the fucosidase is human α -L-fucosidase.

9. The method according to Claim 6, wherein the fucosidase and sialidase are of human origin.
10. The method according to Claim 3, wherein the sialidase is a recombinant enzyme.
11. The method according to Claim 5, wherein the fucosidase is a recombinant enzyme.
12. The method according to Claim 1, wherein the anti-adhesion enzyme is modified to bring the pH requirement for the optimal activity of the enzyme to within the range of pH 7-7.4.
13. The method according to Claim 1, wherein the anti-adhesion enzyme is modified to eliminate the production thereby of sites for N-linked glycosylation.
14. The method according to Claim 1, wherein the anti-adhesion enzyme is chemically conjugated to polyethylene glycol.
15. The method according to Claim 1, wherein the therapeutic efficacy of the anti-adhesion enzyme is monitored by assay of the patient's plasma by means for detecting the extent of any inflammatory response.
16. The method according to Claim 1, wherein the mammal is a human.

1 / 2

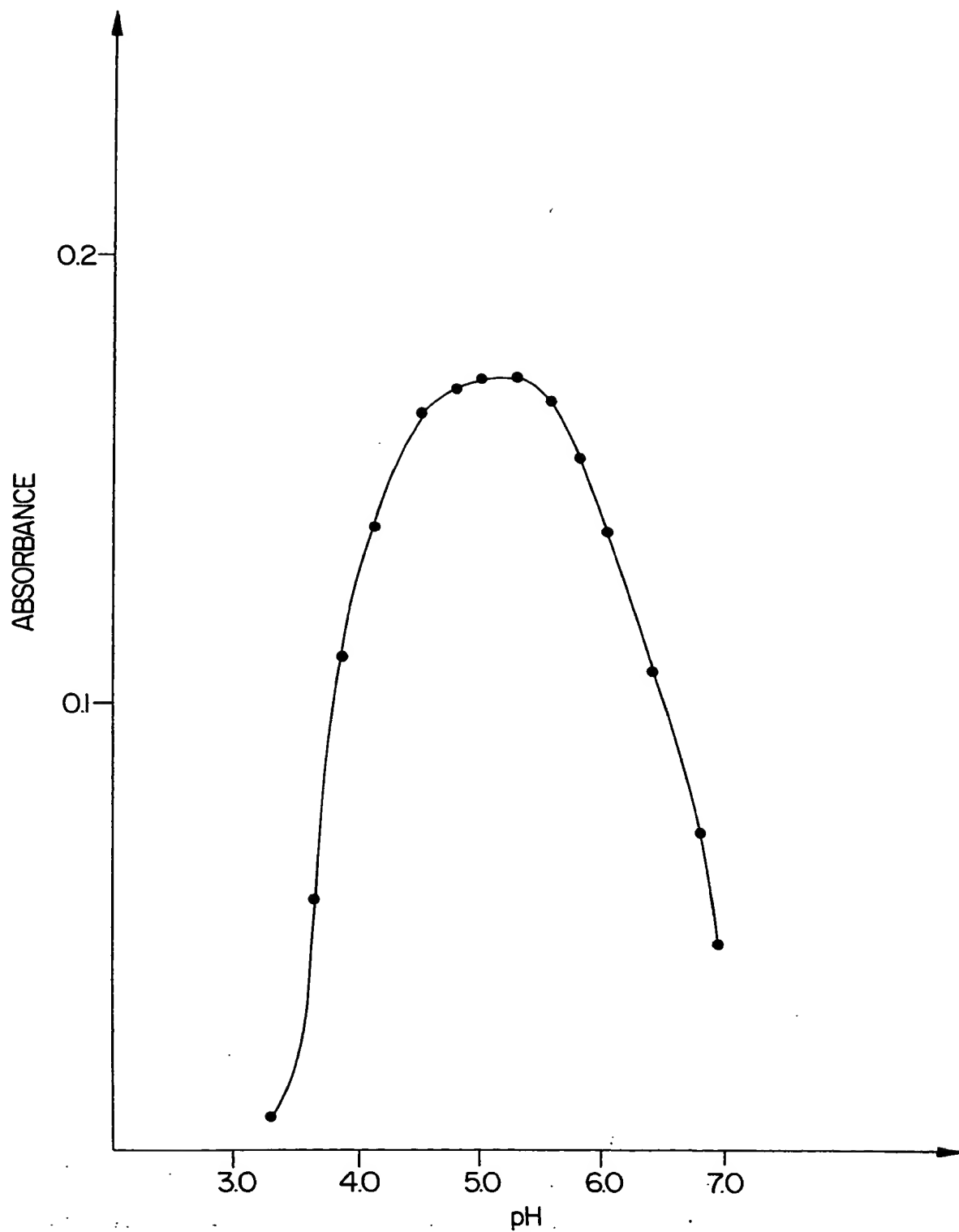


FIG. 1

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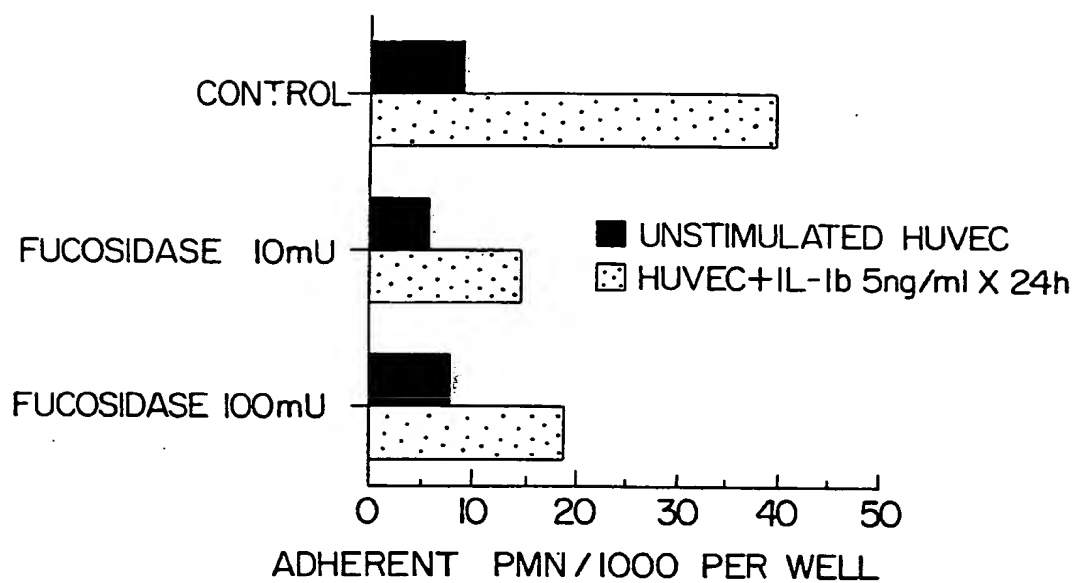


FIG. 2

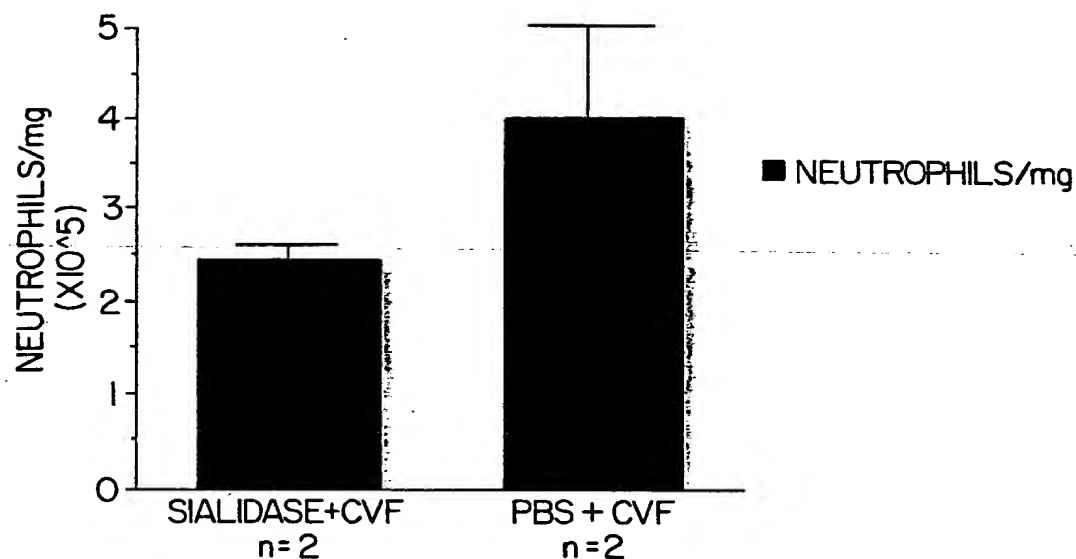


FIG. 3

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/12464

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : A61K 37/54; C12N 9/24

US CL : 514/2, 12; 435/200

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2, 12; 435/200

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	J. Cell Biol., Vol. 115, No. 2, issued October 1991, Zhou et al., "The Selectin GMP-140 Binds To Sialylated, Fucosylated Lactosaminoglycans On Both Myeloid And Nonmyeloid Cells", pages 557-564, see entire document.	<u>1-4</u> 1-16
Y	Proc. Natl. Acad. Sci. USA, Vol. 88, issued February 1991, Tiemeyer et al., "Carbohydrate Ligands For Endothelial-Leukocyte Adhesion Molecule 1", pages 1138-1142, see entire document.	1-16
Y	US, A, 4,904,584 (SHAW, G.) 27 February 1990, see entire document.	1-16



Further documents are listed in the continuation of Box C.



See patent family annex.

Special categories of cited documents:	
A document defining the general state of the art which is not considered to be part of particular relevance	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
E earlier document published on or after the international filing date	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	*G* document member of the same patent family

Date of the actual completion of the international search

08 MARCH 1994

Date of mailing of the international search report

MAR 21 1994

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INTERNATIONAL SEARCH REPORT

 International application N.
 PCT/US93/12464

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Biochem. Biophys. Res. Commun., Vol. 164, No.1, issued 16 October 1989, Occhiodoro et al., "Human α -L-Fucosidase: Complete Coding Sequence From cDNA Clones", pages 439-445, see entire document.	1-16
Y	The Lancet, Vol. 2, issued 04 October 1986, Taylor et al., "Enzyme Replacement In Nervous Tissue After Allogenic Bone-Marrow Transplantation For Fucosidosis In Dogs", pages 772-774, see entire document.	1-16
Y	Biochem. Biophys. Res. Commun., Vol 172, No. 1, Allen et al., "Metabolic Correction Of Fucosidosis Lymphoid Cells By Galaptin- α -L-Fucosidase Conjugates", pages 335-340, see entire document.	1-16
Y	Biochem. J., Vol. 264, issued 1989, Fisher et al., "Isolation And Sequence Analysis Of A cDNA Encoding Rat Liver α -L-Fucosidase", pages 695-701, see entire document.	1-16
Y	Biochim. Biophys. Acta, Vol. 578, issued 1979, Savoca et al., "Preparation Of A Non-Immunogenic Arginase By The Covalent Attachment Of Polyethylene Glycol", pages 47-53, see entire document.	1-16
Y	J. Biol. Chem., Vol. 250, No. 18, issued 25 September 1975, Alhadeff et al., "Human Liver α -L-Fucosidase Purification, Characterization, And Immunochemical Studies", pages 7106-7113, see entire document.	1-16
Y	J. Lab. Clin. Med., Vol. 79, No. 1, Zielke et al., "Fucosidosis: Diagnosis By Serum Assay Of α -L-Fucosidase", pages 164-169, see entire document.	1-16
Y	P. H. POUWELS et al., "Cloning Vectors, A Laboratory Manual", published 1985, by Elsevier, Amsterdam, pages VIII-1 to VIII-3 and VIII-A-b-ii-4. see entire document.	1-16
Y	J. D. WATSON, "Molecular Biology Of The Gene", Third Edition, Benjamin/Cummings Publ. Co., Inc., Menlo Park, California, page 313, see entire document.	1-16
Y	Mol. Gen. Genet., Vol. 226, issued 1991, Rothe et al., "The Sialidase Gene From <i>Clostridium septicum</i> : Cloning, Sequencing, Expression in <i>Escherichia coli</i> And Identification Of Conserved Sequences In Sialidases And Other Proteins", pages 190-197, see entire document.	1-16

INTERNATIONAL SEARCH REPORT

International application N .
PCT/US93/12464

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,675,285 (CLARK ET AL.), 23 June 1987, see entire document.	1-16
Y	Biochem. Biophys. Res. Commun., Vol. 163, No. 3, issued 29 September 1989, Tsuji et al., "Molecular Cloning Of A Full Length cDNA For Human α -N-Acetylgalactosaminidase (α -Galactosidase B)", pages 1498-1504, see especially page 1498.	1-4, 6, 7, 9-16

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application N .

PCT/US93/12464

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS - USPAT, JPOABS

Dialog - files 5, 35, 73, 155, 203, 351, 357, and 144,

EMBL_NEW, GenBank 80, GenBank-NEW, N-GenSeq 13, UEMBL 37_80, VectorBank 6.4

Search terms icam-1, clam-1, fucoisidase?, fucoisylate?, sialylate?, sialidase?, inhibit?, adhesion,
fucoisidosis, inflamm?

